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The Kinetic Equation for the Chloride Transport Cycle of Band 3

A ^{35}Cl AND ^{37}Cl NMR STUDY*

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The nature of a transmembrane transport process depends largely on the identity of the reaction that is rate-limiting in the transport cycle. The one-for-one exchange of two chloride ions across the red cell membrane by band 3 can be decomposed into two component reactions: 1) the binding and dissociation of chloride at the transport site, and 2) the translocation of bound chloride across the membrane. The present work utilizes ^{35}Cl NMR and ^{37}Cl NMR to set lower limits on the rates of chloride binding and dissociation at the saturated inward- and outward-facing band 3 transport sites ($\geq 10^5$ events site^{-1} s^{-1} in all cases). At both 0–3 and 37 °C, the NMR data specify that chloride binding and dissociation at the saturated transport sites are not rate-limiting, indicating that translocation of bound chloride across the membrane is the slowest step in the overall transport cycle.

Using these results, it is now possible to describe many features of the kinetic equation for the ping-pong transport cycle of band 3. This transport cycle can be decomposed into two half-reactions associated with the transport of two chloride ions in opposite directions across the membrane, where each half-reaction is composed of sequential binding, translocation, and dissociation events. One half-reaction contains the rate-limiting translocation event that controls the turnover of the transport cycle; in this half-reaction, translocation must be slower than binding and dissociation. The other half-reaction contains the non-rate-limiting translocation event that in principle could be faster than binding or dissociation. However, when the following sufficient (but not necessary) condition is satisfied, both translocation events are slower than binding and dissociation: if the non-rate-limiting translocation rate is within a factor of 10^2 (0–3 °C) or 2 (37 °C) of the overall turnover rate, then translocation is rate-limiting in each saturated half-reaction. Thus, even though chloride appears to migrate through a channel that leads from the transport site to solution, the results support a picture in which the binding, dissociation, and channel migration events are rapid compared to the translocation of bound chloride across the membrane. In this case, chloride binding to the transport site can be described by a simple dissociation constant ($K_D = k_{OFF}/k_{ON}$) rather than by a Michaelis-Menten constant ($K_M = (k_{OFF} + k_{TRANSLOCATION})/k_{ON}$).

Band 3, which catalyzes the one-for-one exchange of monovalent anions across the red cell membrane, is one of a wide variety of transport proteins found in biological systems. These proteins, despite their diversity, must share certain important features due to their similar functions. In particular, any transport protein that possesses transport site(s) must also possess a transport cycle that can be decomposed into at least three fundamental events: 1) binding of substrate to the transport site(s), 2) translocation of bound substrate across the membrane, and 3) dissociation of substrate from the transport sites. The relative rates of binding, translocation, and dissociation are particularly important because they largely determine the nature of the transport cycle. In many discussions of transport proteins, it is assumed that binding and dissociation of substrates are rapid relative to translocation. This assumption appears reasonable, and it greatly simplifies mathematical models. However, it is impossible to rule out *a priori* a scheme in which binding or dissociation is rate-limiting in the transport cycle. In fact, transport proteins may contain channels leading to transport sites in the interior of the membrane (1), and the diffusion of substrate through such channels could in principle be quite slow.

Substrate channels have been proposed to exist in the band 3 transport unit (1), so it is particularly fortunate that in this system ^{35}Cl and ^{37}Cl NMR can be used to investigate the migration of substrate chloride ion between the transport site and the bulk solution (2, 3). The physical basis of the NMR technique is the large difference in the spectral widths of bound and free chloride; the spectral width of chloride bound to a macromolecule is typically $\geq 10^4$ times larger than the spectral width of solution chloride. As a result, when solution chloride visits a macromolecular binding site sufficiently rapidly, the solution chloride NMR resonance can be measurably broadened. When broadening (termed the line broadening) is observed, it contains information on the rate of chloride migration between the binding site and the bulk solution, in addition to information on the structure and motions of the site (2, 3). The present paper focuses on both the inward- and outward-facing conformations of the band 3 transport site at both 1) the experimentally important temperatures of 0–3 °C, where the bulk of literature chloride transport experiments have been conducted, and 2) the physiological temperature of 37 °C. In each case, a lower limit is placed on the rate of chloride exchange between the transport site and the solution. The resulting lower limits are rapid compared to the known turnover rate of the chloride transport cycle; thus, the rate-limiting step in the transport cycle is the translocation of bound chloride across the membrane rather than a binding or dissociation step. It should be emphasized that this result is

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independent of any model for the transport cycle; however, a wide variety of existing evidence suggests that anion transport by band 3 proceeds via a ping-pong mechanism (4) (reviewed in Refs. 5–7; also see the immediately preceding paper (8)). Thus, a kinetic equation is presented for a ping-pong model of the chloride transport cycle; it is now possible to describe many of the interesting rates in this kinetic equation.

EXPERIMENTAL PROCEDURES

Materials—Freshly outdated human blood (packed red cells) was a kind gift of the Los Angeles Chapter of the American Red Cross. Human carbonic anhydrase B was from Sigma. All other chemicals were reagent grade or better, as previously described (3, 9).

Sample Preparation—NMR samples of leaky red cells ghosts (3), sonicated ghosts (9), crushed ghosts (9), and sealed right-side-out vesicles (ROV[1] (9)) were prepared on ice exactly as previously described using 10-mm sample tubes. In order to determine the $^{35}Cl^-$ or $^{37}Cl^-$ line broadening due to band 3 transport sites, the same volume of H_2O or DNDS stock solution was added to identical samples. The transport site line broadening is given by the line broadening inhibited by 1 mM DNDS and is also termed the DNDS-sensitive line broadening (3). As previously described (3, 9), inward- and outward-facing transport sites were resolved at 3 °C by comparing the line broadening of leaky systems (ghosts, sonicated ghosts) with that of external-site systems (intact red cells, crushed ghosts, and sealed ROV). Similarly inward- and outward-facing transport sites were resolved at 37 °C by comparing the line broadening of sonicated ghosts with that of crushed ghosts.

^{35}Cl and ^{37}Cl NMR Spectroscopy—Spectra were obtained at 3 °C or 37 °C on a JEOL FX-90Q spectrometer (^{35}Cl = 8.8 MHz, ^{37}Cl = 7.3 MHz). The standard spectral parameters used in acquisition of the $^{37}Cl^-$ spectra were the same as those previously described for $^{35}Cl^-$ (3), except that more pulses (5,000–10,000 total pulses) were accumulated due to the lower sensitivity of ^{37}Cl NMR spectroscopy for natural abundance chloride. Total accumulation times were 15–30 min. All spectra that were used to determine a single $^{35}Cl^-/^{37}Cl^-$ line broadening ratio were accumulated consecutively without interruption, using the same number of pulses for both $^{35}Cl^-$ and $^{37}Cl^-$ spectra. The line broadening ratios were determined by obtaining $^{35}Cl^-$ spectra, then $^{37}Cl^-$ spectra (or vice versa), for the same set of six samples: three with 1 mM DNDS and three without 1 mM DNDS.

NMR Sample Analysis—Total membrane protein was determined for NMR samples using the modified (10) Lowry protein assay (11). $^{35}Cl^-$ and $^{37}Cl^-$ line broadenings were normalized to 1 mg/ml total membrane protein as previously described (3).

Calculation of the $^{35}Cl^-/^{37}Cl^-$ Line Broadening Ratio—The line broadenings for each set of three identical samples were averaged to yield the mean line broadening with or without 1 mM DNDS. Then the transport site line broadening (DNDS-sensitive line broadening (3)) was calculated by subtraction of the average line broadenings for samples with and without 1 mM DNDS. The low-affinity site line broadening was simply the line broadening observed in the presence of 1 mM DNDS (DNDS-insensitive line broadening (3)). Finally, the transport site or low-affinity site $^{35}Cl^-/^{37}Cl^-$ line broadening ratio was calculated from the transport site $^{35}Cl^-$ and $^{37}Cl^-$ line broadenings or from the low-affinity site $^{35}Cl^-$ and $^{37}Cl^-$ line broadenings, respectively.

Statistics—For each membrane system the $^{35}Cl^-/^{37}Cl^-$ line broadening ratio was determined three or more times from separate experiments using different membrane batches. These line broadening ratios were then averaged and are presented as means ± 1 S.D.

RESULTS

The Kinetic Information Contained in the $^{35}Cl^-$ or $^{37}Cl^-$ Line Broadening—The exchange of chloride between a binding site and solution can be slow, intermediate, or rapid on the chloride NMR time scale. For slow exchange the $^{35}Cl^-$ or $^{37}Cl^-$ line broadening exactly specifies the exchange rate, while for intermediate and rapid exchange the line broadening places a lower limit on the exchange rate (Appendix[2] and (2)).

In the slow exchange case the kinetics of both binding to and dissociation from a site can be precisely quantitated. The slow exchange case occurs when the lifetime of chloride in the site is much longer than the NMR time scale defined by the transverse relaxation time (T_2) of chloride in the site (the same condition must be satisfied by all three of the chloride NMR transitions). Satisfaction of this condition *guarantees* that a single binding event is sufficient to completely dephase the transverse magnetization contributed by a solution chloride ion. In this limit the $^{35}Cl^-$ or $^{37}Cl^-$ line broadening is given by (Appendix)

$$\delta \begin{cases} = \\ < \\ \ll \end{cases} k_{OFF}p_B/\pi \quad \begin{matrix} \text{slow exchange} \\ \text{intermediate exchange} \\ \text{rapid exchange} \end{matrix} \qquad (1)$$

where k_{OFF} is the rate constant for chloride dissociation from the site and $p_B = ([ECl]/[Cl^-]_T)$ is the fraction of the total chloride that is bound to the site. The equality in Equation 1 holds only when the line broadening is due to single binding events. In the intermediate and rapid exchange cases, the line broadening is due to multiple visits by each solution chloride ion to binding sites; thus, the line broadening yields a lower limit on the exchange rate and the equality changes to one of the indicated conditions.

Equation 1 can be further specified at limiting total chloride concentrations, assuming that the stoichiometric concentration of sites ($[E]_T$) and the chloride dissociation constant ($K_D = k_{OFF}/k_{ON}$) are known. When the site is far from saturation ($[Cl^-]_T \ll K_D$), the binding reaction controls the exchange rate and the line broadening becomes (Appendix)

$$\delta \begin{cases} = \\ < \\ \ll \end{cases} [E]_T k_{ON}/\pi \quad \begin{matrix} \text{slow exchange} \\ \text{intermediate exchange} \\ \text{rapid exchange} \end{matrix} \qquad (2)$$

or, when $[Cl^-]_T \gg K_D$, the dissociation reaction controls the exchange rate and is the line broadening becomes (Appendix)

$$\delta \begin{cases} = \\ < \\ \ll \end{cases} \frac{[E]_T k_{OFF}}{[Cl^-]_T \pi} \quad \begin{matrix} \text{slow exchange} \\ \text{intermediate exchange} \\ \text{rapid exchange} \end{matrix} \qquad (3)$$

Thus, the $^{35}Cl^-$ or $^{37}Cl^-$ line broadening at the very least can be used to place lower limits on k_{ON} and k_{OFF}, while under the most favorable conditions these rate constants can be specified exactly.

The maximum amount of kinetic information can be obtained from the $^{35}Cl^-$ or $^{37}Cl^-$ line broadening only when a site has been classified as a slow, intermediate, or rapid exchange site. This classification becomes possible when the $^{35}Cl^-/^{37}Cl^-$ line broadening ratio is known. For a site in the slow exchange limit, the line broadening is determined solely by the rate of chloride exchange between the site and solution. This rate is essentially the same for $^{35}Cl^-$ and $^{37}Cl^-$ because the kinetic isotope effect is negligible for such relatively heavy ions. As a result, the $^{35}Cl^-/^{37}Cl^-$ line broadening ratio is unity (Appendix):

$$^{35}\delta/^{37}\delta \begin{cases} = 1 & \text{slow exchange} \\ >1, <1.6 & \text{intermediate exchange} \\ = 1.6 & \text{rapid exchange} \end{cases} \qquad (4)$$

[1] The abbreviations used are: ROV, right-side-out vesicles; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate.

[2] Portions of this paper (including Appendix and Table AI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-0941, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

In the rapid exchange limit, the line broadening is independent of the rate of chloride exchange but does depend on the characteristics of the site and on the nuclear electric quadrupole moment. The quadrupole moments of the ^{35}Cl and ^{37}Cl nuclei are known and are significantly different such that the square of their ratio, which determines the line broadening ratio, is 1.6. The intermediate exchange case falls between the extremes of slow and rapid exchange; in this event, $1 < {}^{35}\delta/{}^{37}\delta < 1.6$ (Equation 4). In short, a combination of ^{35}Cl NMR and ^{37}Cl NMR can yield a significant amount of information on the kinetics of chloride exchange between a binding site and solution; moreover, this information is difficult to obtain by any other means.

Chloride Binding and Dissociation Rates for Band 3 Transport Sites at 3 °C—The $^{35}Cl^-$ line broadening due to band 3 transport sites can be resolved into two components that arise from the inward- and outward-facing sites, respectively (9). Each component can be used to determine the lower limit on the rate of binding and dissociation events at the transport sites, using the relationship $k_{OFF} \geq \pi\delta/p_B$ (Equation 1).

For the outward-facing transport site. $^{35}\delta = 1.6 \pm 0.2$ Hz at 3 °C (Table I). Calculation of the fraction of total chloride bound to the outward-facing transport site (p_B) requires knowledge of both 1) the distribution of transport sites between the inward- and outward-facing conformations, and 2) the microscopic chloride dissociation constant for the outward-facing transport site. Neither of these quantities is presently known; however, an upper limit can be placed on p_B since there is at most one occupied outward-facing transport site/band 3 monomer. Thus, $p_B \geq 1.2 \times 10^{-5}$ (Table I). Use of this value in Equation 1 yields $k_{OFF} \geq (4.2 \pm 0.5) \times 10^6$ s^{-1}, which is equivalent to the lower limit on the rate of chloride dissociation from the saturated outward-facing transport site. Since chloride binding and dissociation are assumed to have reached kinetic steady state, the rate of binding events at the saturated outward-facing transport site must also equal k_{OFF} and therefore must satisfy the same condition (on-rate) $\geq (4.2 \pm 0.5) \times 10^5$ s^{-1}. In contrast, the turnover rate of the band 3-catalyzed chloride transport cycle is only 430 s^{-1} at 0 °C and saturating chloride concentration (12); thus, the chloride binding and dissociation reactions at the saturated outward-facing transport site are each at least 2 orders of magnitude too fast to be rate-limiting in the transport cycle.

For the inward-facing transport site $^{35}\delta = 0.7 \pm 0.2$ Hz at 3 °C, and the same calculation outlined for the outward-facing transport site yields the lower limit for the k_{OFF} of this site (Table I). The result, $k_{OFF} \geq (1.8 \pm 0.4) \times 10^5$ s^{-1}, indicates that the rates of chloride binding and dissociation at the saturated inward-facing transport site are again at least 2 orders of magnitude faster than the turnover rate. Thus, the rate-limiting step in the chloride transport cycle is not a binding or dissociation event at either the inward- or outward-facing band 3 transport site.

The $^{35}Cl^-/^{37}Cl^-$ Line Broadening Ratio for Band 3 Transport Sites at 3 °C—An examination of the $^{35}Cl^-/^{37}Cl^-$ line broadening ratio for the inward- and outward-facing transport sites further specifies the rates of chloride exchange between the sites and solution. Moreover, the line broadening ratio indicates whether or not the transport site line broadening is sensitive to changes in the exchange rate.

The outward-facing transport sites can be selectively observed using crushed ghosts and sealed right-side-out vesicles (ROV (9)). This site exhibits a line broadening ratio of 1.09–1.12 at 3 °C (Fig. 1) which places the site in or near the slow exchange limit; that is, the transverse magnetization due to a bound $^{35}Cl^-$ or $^{37}Cl^-$ is completely dephased on a time scale rapid compared to the amount of time spent by the ion in the site ($1/k_{OFF}$). The line broadening due to a slowly exchanging site is sensitive to changes in k_{ON} and k_{OFF} (Equation 1); as a result, specific tests of the band 3 transport mechanism are possible (see Ref. 8). In the slow exchange limit the exchange rate can be exactly specified from the line broadening, assuming that the occupied site concentration is known (Equation 1). However, as discussed above, only an upper limit can be placed on the occupied site concentration. Use of this upper limit in the expression $k_{OFF} = \pi\delta/p_B$ yields the same condition that was already determined (Table I): $k_{OFF} \geq (4.2 \pm 0.5) \times 10^5$ s^{-1}. This is simply a restatement of the previous conclusion that at 3 °C the chloride binding and dissociation events at the outward-facing transport site are at least 2 orders of magnitude too fast to be rate-limiting in the transport cycle.

An alternative explanation for the slow exchange transport site seen in the crushed ghost and ROV systems is that the observed transport site line broadening might be leaking out from the internal compartment, which slowly exchange chloride with the internal compartment. However, this explanation is ruled out by two pieces of evidence: 1) the line broadening ratio for the low-affinity sites on leaky or sonicated ghosts is not significantly larger than the line broadening ratio for the low-affinity sites on crushed ghosts or ROV (Fig. 2), and 2) the outward-facing transport sites are also observed in the intact red cell system, where line broadening due to internal sites cannot leak into the external compartment.

Both inward-facing transport sites and outward-facing transport sites are observed in the leaky ghost and sonicated ghost systems (9), and in these systems the $^{35}Cl^-/^{37}Cl^-$ line broadening ratio increases significantly relative to the isolated outward-facing transport site (Fig. 1). Thus, the inward-facing transport sites are significantly closer to the rapid exchange limit than are the outward-facing transport sites. This analysis is supported by the line broadening ratio calculated for the isolated inward-facing transport sites; subtraction of the line broadening due to the outward-facing transport sites (crushed ghosts, ROV) from the line broadening due to both

TABLE I

The lower limit on k_{OFF} for chloride dissociation from the band 3 transport site

$k_{OFF} \geq \pi\delta/p_B$	
Outward-facing transport site	Inward-facing transport site
$\delta = 1.6 \pm 0.2$ Hz[a], 3 °C $= 1.7 \pm 0.2$ Hz[a], 37 °C	$\delta = 0.7 \pm 0.2$ Hz[a] $= 0.5 \pm 0.4$ Hz[a]
[Band 3] = 3.0 μM (monomer)	[Band 3] = 3.0 μM
$[Cl^-]_T$ = 250 mM	$[Cl^-]_T$ = 250 mM
$p_B \leq$ [band 3 monomer]/$[Cl^-]_T$ $= 1.2 \times 10^{-5}$ [b]	$p_B \leq 1.2 \times 10^{-5}$ [b]
$k_{OFF} \geq (4.2 \pm 0.5) \times 10^6$ s^{-1}, 3 °C $\geq (4.5 \pm 0.5) \times 10^6$ s^{-1}, 37 °C	$k_{OFF} \geq (1.8 \pm 0.4) \times 10^5$ s^{-1}, 3 °C $\geq (1.3 \pm 0.8) \times 10^5$ s^{-1}, 37 °C

[a] For 1 mg/ml total ghost protein, 8.8 MHz (3, 9).
[b] Assuming 1 transport site/band 3 monomer.

inward- and outward-facing transport sites (leaky ghosts, sonicated ghosts) yields the line broadening due to inward-facing transport sites. (The line broadenings used in the present calculation are those used to generate Fig. 1.) According to this calculation, the inward-facing transport sites exhibit a $^{35}Cl^-/^{37}Cl^-$ line broadening ratio of 2.3 ± 0.6 at 3 °C, which is larger than the largest possible value of 1.6 for the rapid exchange limit. However, the uncertainty in the measured ratio is large due to the subtraction, so that the ratio is not statistically different from 1.6. This result, as well as the increase in the line broadening ratio observed when the inward-facing transport sites are added to the outward-facing sites (Fig. 1), together indicate that the inward-facing transport sites are in or near the rapid exchange limit. It follows that 1) the line broadening due to the inward-facing transport sites is independent of moderate changes in k_{ON} or k_{OFF}, and 2) the expression $k_{OFF} \gg \pi\delta/p_B$ (Equation 1) can be used to place a strong condition on the exchange rate for the inward-facing sites. Using the lower limit obtained from the ^{35}Cl line broadening (Table I), the exchange rate becomes $k_{OFF} \gg (1.8 \pm 0.4) \times 10^5\ s^{-1}$. Thus, the chloride binding and dissociation events at the saturated inward-facing transport site must be more than 2 orders of magnitude faster than the turnover rate of the chloride transport cycle.

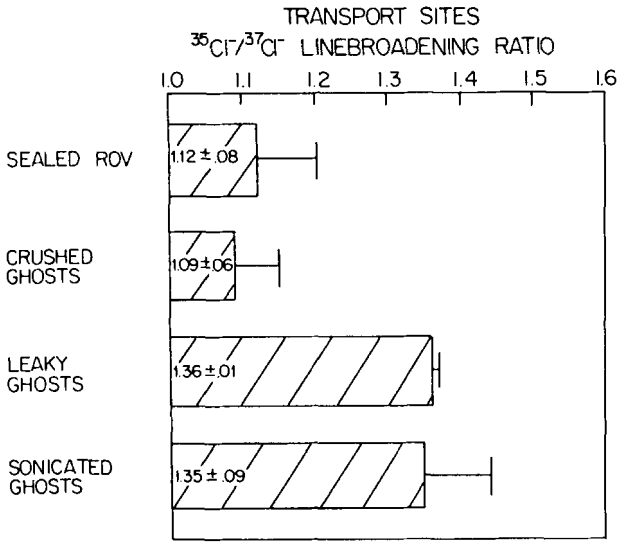

FIG. 1. **Comparison of the $^{35}Cl^-/^{35}Cl^-$ line broadening ratio of inward- and outward-facing band 3 transport sites.** The line broadening ratio of outward-facing transport sites (sealed ROV, crushed ghosts) is compared with the line broadening ratio of both inward- and outward-facing transport sites (leaky ghosts, sonicated ghosts). All samples contained 1–2 mg/ml total membrane protein and 250 mM NH_4Cl, 20% D_2O, 5 mM NaH_2PO_4, pH to 8.0 with NaOH, NH_4OH; the sealed ROV also contained 100 μM $MgSO_4$. Spectra were obtained at 8.8 MHz ($^{35}Cl^-$) or 7.3 MHz ($^{37}Cl^-$) at 3 °C.

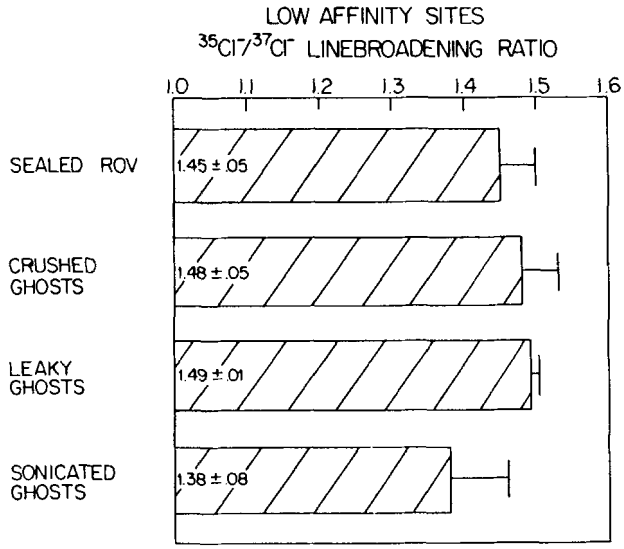

FIG. 2. **Comparison of the $^{35}Cl^-/^{37}Cl^-$ line broadening ratio of inward- and outward-facing low-affinity sites.** The line broadening ratio of outward-facing low-affinity sites (sealed ROV, crushed ghosts) is compared with the line broadening ratio of both inward- and outward-facing low-affinity sites (leaky ghosts, sonicated ghosts). Sample compositions and spectra were as described in the legend to Fig. 1.

The $^{35}Cl^-/^{37}Cl^-$ Line Broadening Ratio for the Low-affinity Chloride Binding Sites at 3 °C—Little is known about the low-affinity chloride binding sites on red cell membranes revealed by ^{35}Cl NMR; their function is undetermined and it is not known whether they are a homogeneous or heterogeneous class of sites. These sites, which are distributed between both sides of the membrane (9), exhibit a macroscopic line broadening ratio of 1.38–1.49 at 3 °C (Fig. 2). No significant difference is observed between the line broadening ratio for outward-facing low-affinity sites (crushed ghosts, ROV) and the line broadening ratio for both inward- and outward-facing low-affinity sites (leaky ghosts, sonicated ghosts, Fig. 2). At the present time it is not possible to determine whether the observed line broadening ratios are due to a homogeneous class of intermediate exchange sites or to heterogeneous classes of slow, intermediate, and/or rapid exchange sites. Moreover, since nothing is known about the number of low-affinity sites, it is not possible to place any limits on the rate of chloride exchange between these sites and solution.

Positive Controls for the $^{35}Cl^-/^{37}Cl^-$ Line Broadening Ratio at 3 °C—Human carbonic anhydrase B possesses a chloride binding site that has previously been shown to be in or near the slow exchange limit by the temperature dependence of the $^{35}Cl^-$ line broadening (13). In the present experiments the human carbonic anhydrase B site exhibits a line broadening ratio of 1.10 ± 0.04 at 3 °C (Fig. 3), thereby confirming the previous conclusion that this site approximates the slow exchange limit.

The line width (Δ) of the solution chloride $^{35}Cl^-$ or $^{37}Cl^-$ resonance is dominated by quadrupole effects; thus, the ratio of these line widths should approach $^{35}\Delta/^{37}\Delta = 1.6$. In the present experiments the observed ratio is 1.49 ± 0.01 at 3 °C (Fig. 3), which is near the theoretically predicted value. Most

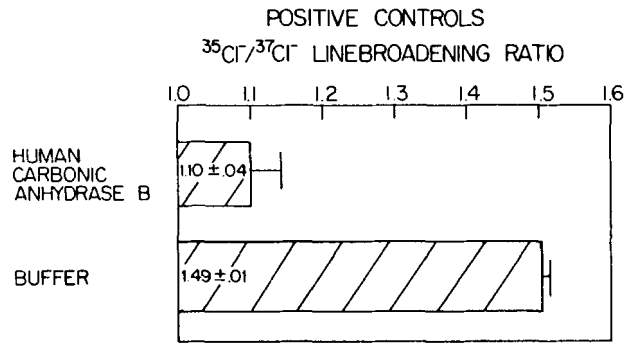

FIG. 3. **Comparison of the $^{35}Cl^-/^{37}Cl^-$ line width ratio and the $^{35}Cl^-/^{37}Cl^-$ line broadening ratio of positive controls.** The line width ratio of buffer-only samples (250 mM NH_4Cl, 20% D_2O, 5 mM NaH_2PO_4, pH to 8.0 with NaOH, NH_4OH) is compared with the line broadening ratio of human carbonic anhydrase B (1 mg/ml in 500 mM NaCl, 20% D_2O, 10 mM NaH_2PO_4, pH 7.4 with NaOH). Spectra were as described in the legend to Fig. 1.

significantly, these positive controls indicate that the slow and rapid exchange limits are easily resolved experimentally (Fig. 3).

The Kinetics of Chloride Binding and Dissociation at the Band 3 Transport Site at 37 °C—For technical reasons, nearly all kinetic studies of chloride exchange by band 3 have been carried out at 0 °C. The present results, obtained at the similar temperature of 3 °C, facilitate the interpretation of the kinetic data and contribute to the development of a kinetic equation at these low temperatures. Although it is highly probable that the mechanism of transport is the same at 0 and 37 °C, it is important to ascertain whether the kinetic conclusions derived at low temperatures can be extended to physiological temperature.

Calculations completely analogous to those previously outlined for 0–3 °C indicate that at 37 °C the rate of chloride dissociation at the outward-facing transport site satisfies $k_{OFF} \geq (4.5 \pm 0.5) \times 10^5\ s^{-1}$, while at the inward-facing transport site $k_{OFF} \geq (1.3 \pm 0.8) \times 10^5\ s^{-1}$ Table I). In the case of saturating chloride concentrations the same lower limits also describe the chloride binding rates.

The observed binding and dissociation events at the outward-facing transport site are faster than the known turnover rate of the saturated chloride transport cycle, which is $8 \times 10^4\ s^{-1}$ at 37 °C (14). Thus, binding and dissociation at the outward-facing transport site do not limit the rate of the transport cycle at physiological temperature. However, at the inward-facing transport site the turnover rate at 37 °C has become comparable to the rates of chloride binding and dissociation, so these rates must be examined more closely.

At 3 °C (see above) binding and dissociation at the inward-facing transport site are in the rapid exchange limit where binding and dissociation are much faster than the calculated lower limit. Since an increase in temperature will generally only increase binding and dissociation rates (barring a structural change in the protein), it is likely that these rates remain much faster than the calculated lower limit at 37 °C. Consistent with this conclusion is the observation that the $^{35}Cl^-$ line broadening due to the inward-facing transport sites is essentially the same at 3 and 37 °C (Table I), which is expected for sites in the rapid exchange limit where line broadening is independent of the rate of chloride exchange between the site and solution. Moreover, the $^{35}Cl^-/^{37}Cl^-$ line broadening ratio for the inward-facing transport site is 2.7 ± 0.5 at 37 °C, indicating that the site is in the rapid exchange limit. Thus, the data require that chloride dissociation from the inward-facing transport site at 37 °C satisfies $k_{OFF} \gg (1.3 \pm 0.8) \times 10^5\ s^{-1}$, guaranteeing that chloride binding and dissociation at the inward-facing transport site are not rate-limiting in the saturated chloride transport cycle. Instead, the rate-limiting step at physiological temperatures is a translocation event.

DISCUSSION

The ^{35}Cl and ^{37}Cl NMR data presented here yield the rate of chloride binding and dissociation at both the inward-facing (E_I) and outward-facing (E_O) band 3 transport sites:

$$Cl_I^- + E_I \underset{\gg 10^5\ s^{-1}}{\overset{\gg 10^5\ s^{-1}}{\rightleftharpoons}} ClE_I \qquad ClE_O \underset{>10^5\ s^{-1}}{\overset{>10^5\ s^{-1}}{\rightleftharpoons}} Cl_O^- + E_O \qquad \text{(I)}$$

where the indicated values are the number of events/saturated transport site/s at 3 and 37 °C (or, equivalently, k_{OFF} for chloride dissociation from the site). At both low temperature and physiological temperature, the chloride binding and dissociation events at both orientations of the saturated transport site are measurably faster than the turnover rate of the saturated chloride transport cycle; thus, the rate-limiting step in the transport cycle is the translocation of bound chloride rather than chloride binding or dissociation. It should be emphasized that these conclusions regarding rate-limiting steps are not dependent on any model for the transport mechanism.

The ping-pong model is now generally accepted as an accurate description of the band 3 transport cycle (4), reviewed in (Refs. 5–7); see also the immediately preceding paper (8). The chloride binding and dissociation rates determined here allow important features of the kinetic equation for a ping-pong transport cycle to be described for the first time:

$$Cl_I^- + E_I \underset{\gg 10^5\ s^{-1}}{\overset{\gg 10^5\ s^{-1}}{\rightleftharpoons}} ClE_I \underset{(8 \times 10^4\ s^{-1},\ 37\ °C)}{\overset{(4 \times 10^2\ s^{-1},\ 0\ °C)}{\rightleftharpoons}} \cdot ClE_O \underset{>10^5\ s^{-1}}{\overset{>10^5\ s^{-1}}{\rightleftharpoons}} Cl_O^- + E_O \qquad \text{(II)}$$

where the translocation of bound chloride is shown to be the rate-limiting step. There are two directions of translocation, and the transport cycle can be considered as the sum of an $I \rightarrow O$ half-reaction with an $O \rightarrow I$ half-reaction. In the rate-limiting half-reaction, the rate of the translocation step must approximately equal the turnover rate of the transport cycle, so that the translocation step in this half-reaction must be slower than chloride binding and dissociation (Equation II). In the non-rate-limiting half-reaction the translocation step could in principle be significantly faster, such that binding or dissociation is rate-limiting for the half-reaction. However, such ambiguity is unlikely at 0–3 °C; assuming that the two translocation rates are within 2 orders of magnitude of each other, the translocation step is rate limiting in both of the half-reactions in the transport cycle (Equation II). Ambiguity is more difficult to exclude at 37 °C, where translocation is required to be rate-limiting in both half-reactions only when both translocation rates are the same order of magnitude as the overall turnover rate (Equation II). However, at both temperatures the data remain consistent with a picture in which translocation events are always slow compared to binding and dissociation events at the saturated transport site.

The kinetic equation is made still more complete by inclusion of channel migration steps for both orientations of the transport site, since recent results indicate that both internal and external solution chloride must migrate through substrate channels to reach the transport site (1).

$$\begin{array}{l} Cl_I^- + \text{CHANNEL}_I + E_I \underset{\gg 10^5\ s^{-1}}{\overset{\gg 10^5\ s^{-1}}{\rightleftharpoons}} Cl\cdot\text{CHANNEL}_I + E_I \underset{>10^5\ s^{-1}}{\overset{\gg 10^5\ s^{-1}}{\rightleftharpoons}} \textit{CHANNEL}_I + \underline{ClE_I} \\ \qquad\qquad \Big\downarrow \begin{matrix}(4 \times 10^2\ s^{-1},\ 0\text{–}3\ °C)\\(8 \times 10^4\ s^{-1},\ 37\ °C)\end{matrix} \Big\uparrow \\ Cl_O^- + \text{CHANNEL}_O + E_O \underset{>10^5\ s^{-1}}{\overset{>10^5\ s^{-1}}{\rightleftharpoons}} Cl\cdot\textit{CHANNEL}_O + E_O \underset{>10^5\ s^{-1}}{\overset{>10^5\ s^{-1}}{\rightleftharpoons}} \text{CHANNEL}_O + \underline{ClE_O} \end{array} \qquad \text{(III)}$$

As indicated by the kinetic equation, the journey by chloride from solution to the transport site can be decomposed into the fundamental steps of 1) channel migration, and 2) binding; note that the rates of chloride binding and dissociation presented here describe the entire journey. The last major undetermined feature of the kinetic equation is the ratio of the in-to-out and out-to-in translocation rate constants. For a ping-pong transporter this ratio partly determines the macroscopically observed dissociation constants of the inward- and outward-facing transport sites, as well as the distribution of transport sites between these two conformations. Thus, additional work is still needed to completely specify the kinetic equation for the band 3 transport cycle.

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Supplementary Material to

THE KINETIC EQUATION FOR THE CHLORIDE TRANSPORT CYCLE OF BAND 3:

A ^{35}Cl AND ^{37}Cl NMR STUDY

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APPENDIX. USE OF THE $^{35}Cl^-/^{37}Cl^-$ LINEBROADENING RATIO TO DETERMINE RATE CONSTANTS FOR CHLORIDE BINDING AND DISSOCIATION.

INTRODUCTION

The $^{35}Cl^-$ and $^{37}Cl^-$ linebroadenings due to chloride binding to an anion binding site are observed only when chloride exchanges sufficiently rapidly between the site and solution; as a result both types of linebroadening contain information on the rate of chloride exchange between these two environments.

Both ^{35}Cl and ^{37}Cl are spin 3/2 nuclei with essentially identical NMR properties so that the same NMR relaxation processes (2,3) are important for both nuclei. However, the quadrupole moments of ^{35}Cl and ^{37}Cl are measurably different, and this difference can be exploited to ascertain whether the chloride exchange between the site and solution is slow or rapid on the NMR timescale. In particular, in the slow exchange limit the linebroadening is insensitive to the difference between the quadrupole moments, while in the rapid exchange limit the linebroadening is sensitive to this difference. As a result, the ratio of the $^{35}Cl^-$ and $^{37}Cl^-$ linebroadenings is unity for slowly exchanging sites but deviates from unity for rapidly exchanging sites. In fact, the $^{35}Cl^-/^{37}Cl^-$ linebroadening ratio can be used to characterize a site as slowly or rapidly exchanging site on the appropriate NMR timescale, and once the characterization has been made the maximum information on chloride exchange rates can be extracted from the linebroadening. For a slowly exchanging site, the linebroadening completely specifies the chloride binding and dissociation rate constants (k_{ON} and k_{OFF}) and the chloride dissociation constant ($K_D = k_{OFF}/k_{ON}$), while for a site in the rapid exchange limit lower limits can be placed on these rate constants.

THE SLOW EXCHANGE LIMIT

A simple derivation will show that a site in the slow exchange limit has a $^{35}Cl^-/^{37}Cl^-$ linebroadening ratio of unity. This limit occurs when each of the three $^{35}Cl^-$ or $^{37}Cl^-$ NMR transitions fulfills the condition

$$\tau_{OFF} = 1/k_{OFF} >> T_{2B_n} \tag{A1}$$

where τ_{OFF} is the length of time a chloride ion typically remains in the site, and T_{2B_n} is the intrinsic NMR relaxation time for the n^{th} transition when chloride is bound to the site (see Appendix II in (3)). In this case, complete dephasing of the transverse magnetization occurs for a bound chloride ion before it can leave the site, and the $^{35}Cl^-$ or $^{37}Cl^-$ linebroadening (δ) for each transition is given by (Appendix II in (3))

$$\delta = k_{OFF}\, p_B/\pi \tag{A2}$$

where p_B is the fraction of total chloride in the sample that is bound to the site. The quantity p_B can be rewritten when the free chloride concentration ($[Cl^-]_F$) is essentially equal to the total chloride concentration ($[Cl^-]_T$):

$$p_B = \frac{[ECl]}{[Cl^-]_T} = \left([E]_T \frac{[Cl^-]_F}{[Cl^-]_F + K_D}\right) \Big/ [Cl^-]_T \cong \frac{[E]_T}{[Cl^-]_T + K_D} \tag{A3}$$

where ECl is bound chloride, $[E]_T$ is the stoichiometric concentration of site E, and K_D is the dissociation constant for chloride at site E. Combining Equations A2 and A3 yields the desired result:

$$\delta = \frac{[E]_T}{\pi} \cdot \frac{k_{OFF}}{[Cl^-]_T + K_D} = \frac{[E]_T}{\pi} \cdot \frac{k_{OFF}}{[Cl^-]_T + (k_{OFF}/k_{ON})} \tag{A4}$$

The quantities k_{ON} and k_{OFF} are essentially the same for $^{35}Cl^-$ and $^{37}Cl^-$ since ions of such relatively large mass exhibit a negligible kinetic isotope effect. Thus, for a given $[E]_T$ and $[Cl^-]_T$ the $^{35}Cl^-/^{37}Cl^-$ linebroadening ratio is simply

$$^{35}\delta/^{37}\delta = 1 \tag{A5}$$

This value of unity is diagnostic for the slow exchange case.

Once a site has been shown by its $^{35}Cl^-/^{37}Cl^-$ linebroadening ratio to be in the slow exchange limit, then (assuming that $[E]_T$ is known) Equation A4 can be used to determine the values of k_{ON} and k_{OFF}. In particular, a plot of δ vs. $[Cl^-]_T$ will yield best-fit values of both k_{ON} and k_{OFF}. It is interesting to note that when the site is far from saturation ($[Cl^-]_T << K_D$), the linebroadening is controlled by the on-reaction (Equation A4):

$$\delta = [E]_T k_{ON}/\pi \tag{A6}$$

In contrast, when the site approaches saturation ($[Cl^-]_T >> K_D$) the off-reaction dominates (Equation A4).

$$\delta = [E]_T\, k_{OFF}/(\pi[Cl^-]_T) \tag{A7}$$

These simple limiting behaviors stem from the fact that in the slow exchange limit the linebroadening is equal to the rate that a chloride in solution becomes bound to the site. When the site is far from saturation, the on-reaction is rate-limiting and controls the binding rate, while when the site is near saturation, the off-reaction is rate-limiting and controls the binding site.

THE RAPID EXCHANGE LIMIT

Here it will be shown that a site in the rapid exchange limit exhibits a $^{35}Cl^-/^{37}Cl^-$ linebroadening ratio of 1.6. This limit occurs when each of the ^{35}Cl or ^{37}Cl NMR transitions fulfills the condition

$$\tau_{OFF}^{-2} >> T_{2B_n}^{-2} \cdot \overline{(\Delta\omega_{\theta,n}^2)} \tag{A8}$$

where $\Delta\omega_{\theta,n}$ is the frequency shift for the n^{th} transition that occurs upon chloride binding, which depends upon the orientation (θ) of the electric-field gradient at the nucleus relative to the direction of the magnetic field. In this limit a chloride ion can visit many sites before complete dephasing of the transverse magnetization occurs, and the $^{35}Cl^-$ or $^{37}Cl^-$ linebroadening of the n^{th} transition is given by (Appendix II in (3))

$$\delta = \frac{p_B}{\pi k_{OFF}}\left(\frac{1}{T_{2B_n}^2} + \overline{(\Delta\omega_{\theta,n}^2)} + \frac{k_{OFF}}{T_{2B_n}}\right) \tag{A9}$$

This expression can be simplified because Equation A8 guarantees that $k_{OFF}/T_{2B_n} >> T_{2B_n}^{-2}$; thus the linebroadening becomes

$$\delta = \frac{p_B}{\pi k_{OFF}}\left(\overline{(\Delta\omega_{\theta,n}^2)} + \frac{k_{OFF}}{T_{2B_n}}\right) \tag{A10}$$

It is important to examine $\overline{(\Delta\omega_{\theta,n}^2)}$, which can be explicitly written

$$\overline{(\Delta\omega_{\theta,n}^2)} = \begin{cases} \overline{(\sigma^2)} & \text{for } +\tfrac{1}{2} \rightarrow -\tfrac{1}{2} \text{ transition} \\ \overline{((\sigma \pm \frac{\pi}{4}\chi(3\cos^2\theta - 1)S)^2} & \text{for } -\tfrac{1}{2} \rightarrow -\tfrac{3}{2} \text{ transition and } +\tfrac{3}{2} \rightarrow +\tfrac{1}{2} \text{ transition} \end{cases} \tag{A11}$$

where σ is the frequency shift of the central transition that occurs upon binding, χ is the quadrupole coupling constant, and S is the order parameter associated with the motional averaging of the nuclear electric quadrupolar interaction experienced by the bound chloride during its lifetime at the site. The quantity σ complicates the analysis of the linebroadening ratio because the central frequency shift is independent of the quadrupole moment while the quantities χ and T_{2B_n} are each dependent upon the quadrupole moment. Fortunately, in the experimental system employed here, the σ term contributes at most 0.5% of the observed linebraodening due to band 3 transport sites (Table AI) so that this term can be neglected and the linebroadening becomes

$$\delta = \begin{cases} \frac{p_B}{\pi T_{2B_n}} & \text{for } +\tfrac{1}{2} \quad -\tfrac{1}{2} \text{ transition} \\ \frac{p_B}{\pi k_{OFF}}\left(\overline{(\frac{\pi}{4}\chi(3\cos^2\theta - 1)S)^2} + \frac{k_{OFF}}{T_{2B_n}}\right) & \text{for } -\tfrac{1}{2} \rightarrow -\tfrac{3}{2} \text{ transition and } +\tfrac{3}{2} \rightarrow +\tfrac{1}{2} \text{ transition} \end{cases} \tag{A12}$$

or, when the θ term is averaged over a uniform distribution of θ

$$\delta = \begin{cases} \dfrac{P_B}{\pi T_{2B_n}} & \text{for } +\tfrac{1}{2} \leftrightarrow -\tfrac{1}{2} \text{ transition} \\ \dfrac{P_B}{\pi k_{OFF}} \left(\dfrac{(\chi\pi S)^2}{20} + \dfrac{k_{OFF}}{T_{2B_n}} \right) & \text{for } -\tfrac{1}{2} \leftrightarrow -\tfrac{3}{2} \text{ transition and } +\tfrac{3}{2} \leftrightarrow +\tfrac{1}{2} \text{ transition} \end{cases} \quad \text{(A13)}$$

The quantities χ and T_{2B_n} each are related to the quadrupole moment (Q):

$$\chi = C_1 Q \quad \text{and} \quad 1/T_{2B_n} = C_2 Q^2 \quad \text{(A14)}$$

where C_1 and C_2 are constants that are the same for ^{35}Cl and ^{37}Cl (2,3), assuming that the dependence of T_{2B_n} on the NMR frequency is not important because the ^{35}Cl and ^{37}Cl NMR frequencies are quite similar (8.8 MHz and 7.3 MHz, respectively, in the present study). Noting that the kinetic isotope effect is small so that the values of k_{OFF} and P_B are the same for $^{35}Cl^-$ and $^{37}Cl^-$, the desired final result is obtained by combining Equations A12 and A13:

$$^{37}\delta/^{37}\delta = (^{35}Q)^2/(^{37}Q)^2 = 1.6 \quad \text{(A15)}$$

This value is measurable larger than unity and thus may be used to distinguish the rapid exchange limit from the slow exchange limit.

In general, k_{ON} and k_{OFF} cannot be determined from the linebroadening in the rapid exchange limit unless the quantities P_B, χ, S, and T_{2B_n} are all known. Yet a lower limit still can be placed on k_{ON} and k_{OFF} because Equation A10 can be rewritten as

$$\delta = (k_{OFF} P_B/\pi) \left\{ (\overline{\Delta\omega^2_{n,\theta}}) \tau^2_{OFF} + \tau_{OFF}/T_{2B_n} \right\} \quad \text{(A16)}$$

and from Equation A8 the bracketed quantity is $\ll 1$. Thus, in the rapid exchange limit

$$\delta \ll k_{OFF} P_B/\pi . \quad \text{(A17)}$$

By analogy with Equations A6 and A7, Equation A16 can be rewritten for the case $[Cl^-] \ll K_D$ where the site is far from saturation

$$\delta \ll [E]_T k_{ON}/\pi \quad \text{(A18)}$$

or, for the case $[Cl^-]_T \gg K_D$ where the site is near saturation

$$\delta \ll [E]_T k_{OFF}/([Cl^-]_T \pi) \quad \text{(A19)}$$

These equations provide lower limits on k_{OFF} and k_{ON} when $[E]_T$ and $[Cl^-]_T$ are known. Such lower limits can be quite useful for certain applications as in the determination of the rate-limiting step in a reaction that involves chloride binding and/or dissociation.

TABLE AI

Sample conditions = 250 mM Cl^-, 1 mg/ml total ghost protein (≈ 3 μm band 3), 3° C

P_B for transport site	≤ [band 3 monomers]/$[Cl^-]_T$ = 3 μM/250 mM = 1×10^{-5}
τ_{OFF}	≤ 1×10^{-5} sec (see text)
σ	≤ 1000 ppm for ^{35}Cl, ^{37}Cl
	≤ 8800 Hz for 8.8 MHz NMR frequency
δ(CALCULATED)	$= \dfrac{P_B \tau_{OFF} \sigma^2}{\pi} \leq 10^{-2}$ Hz
δ(OBSERVED)	= 2Hz
$\dfrac{\delta(\text{OBSERVED})}{\delta(\text{CALCULATED})}$	≥ 200